Exposure-dose-response of *Tellina deltoidalis* to contaminated estuarine sediments

3. Selenium spiked sediments

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Abstract

The metalloid selenium is an essential element which at slightly elevated concentrations is toxic and mutagenic. In Australia the burning of coal for power generation releases selenium into estuarine environments where it accumulates in sediments. The relationship between selenium exposure, dose and response was investigated in the deposit feeding, benthic, marine bivalve *Tellina deltoidalis*. Bivalves were exposed in microcosms for 28 days to individual selenium spiked sediments, 0, 5 and 20 µg/g dry mass. *T. deltoidalis* accumulated selenium from spiked sediment but not in proportion to the sediment selenium concentrations. The majority of recovered subcellular selenium was associated with the nuclei and cellular debris fraction, probably as protein bound selenium associated with plasma and selenium bound directly to cell walls. Selenium exposed organisms had increased biologically detoxified selenium burdens which were associated with both granule and metallothionein like protein fractions, indicating selenium detoxification. Half of the biologically active selenium was associated with the mitochondrial fraction with up to 4 fold increases in selenium in exposed organisms. Selenium exposed *T. deltoidalis* had significantly reduced GSH:GSSG ratios indicating a build-up of oxidised glutathione. Total antioxidant capacity of selenium exposed *T. deltoidalis* was significantly reduced which corresponded with increased lipid peroxidation, lysosomal destabilisation and micronuclei frequency. Clear exposure – dose – response relationships have been demonstrated for *T. deltoidalis* exposed to selenium spiked sediments, supporting its suitability for use in selenium toxicity tests using sub-lethal endpoints.

Keywords: Biomarkers, subcellular selenium, oxidative stress, lysosomes, lipid peroxidation, micronuclei, bivalve.

1 Introduction

Selenium is an essential element within a fairly narrow concentration range, above which it is both mutagenic and toxic and below which selenium deficiency occurs (Hodson, 1988; Hoffman, 2002). Selenium studies which examined selenium dietary requirements, in the trout *Salmo gairdneri* showed that plasma glutathione peroxidase homeostasis was maintained at intakes of up to 1.25 µg/g dry food and toxicity occurred at 13 µg/g dry food. The authors speculated that dietary concentrations in excess of 3 µg/g in dry food over long time periods might be toxic. (Hilton et al., 1980; Hodson et al., 1980; Hodson and Hilton, 1983; Hicks et al., 1984). Eisler (2000) and Puls (1994) have reported similar responses in other fish species,

birds and mammals in relation to selenium dietary requirements, deficiency, and toxicity. Selenium is released into aquatic environments through industrial activity such as metal smelting, overflow and leaching from ash dams and stack emissions associated with coal fired power stations and through sewage effluent (Davies and Linkson, 1991; Peters et al., 1999a). Sediments contain most of the total estuarine selenium inventory because of sorption and/or precipitation mechanisms (Peters et al., 1999a). Selenium biotransformation, bioaccumulation, and transfer through both sediment and water column foodwebs constitute major biogeochemical pathways in aquatic ecosystems (Lemly, 1999; Fan et al., 2002; Hamilton, 2004; Luoma and Rainbow, 2008; Maher et al., 2010). To acquire sufficient essential elements from environments with low ambient concentrations, aquatic organisms have evolved highly efficient uptake mechanisms, coupled with detoxification storage and excretion strategies (Phillips and Rainbow, 1989). Selenium accumulation by sediment dwelling deposit feeding bivalves may be from the interstitial water, sediment ingestion or from food (Luoma and Rainbow, 2005). The route of uptake may influence the organism's metal handling and therefore its toxicity (Rainbow, 2007). Physiological effects and toxicity of metals strongly depend on their intracellular localisation and binding to organelles and ligands (Sokolova et al., 2005) and selenium appears to be bound and incorporated differently according to the selenium species (Ewan, 1989; Burk, 1991; Hortensia et al., 2006).

To understand the fate and effects of such toxicants in aquatic environments the causal relationships between contaminant exposure, internal dose and associated biological effects need to be established (Widdows and Donkin, 1992; Adams et al., 2011). The evaluation of contaminant exposure, uptake and ecotoxicological effects is now an essential component of sediment quality assessment in Australia (Simpson et al., 2005) and toxicity data for local species along with suitable routine test protocols is necessary to develop relevant local exposure dose response toxicity guidelines. The current developments in ecotoxicological assessment are moving to the evaluation of sub lethal endpoints for determining toxicant guideline exposure concentrations. To this end the development of biomarkers of exposure and effect for application in environmental assessment have been progressively developed and refined for a range of toxicants and aquatic species (Cajaraville et al., 2000; Adams, 2001; van der Oost et al., 2003; Galloway et al., 2004; Moore et al., 2004; Farmer, 2006; Batley et al., 2007; Damiens et al., 2007; Hagger et al., 2009; Taylor and Maher, 2010). Biomarker measurements can provide evidence that organisms have been exposed to contaminants at levels that exceed their detoxification and repair capacity establishing links between toxicant exposure and ecologically relevant effects (Koeman et al., 1993). Proteins contain the

majority of selenium in organisms and of the known selenoproteins, cellular and plasma glutathione peroxidase, which is involved in redox metabolism, has the highest selenium content (Burk, 1991; Fan et al., 2002). The oxidative system has been shown to be sensitive to selenium through perturbations in the glutathione cycle (Hoffman, 2002). Lysosomes are involved in contaminant sequestration and are also susceptible to oxidative damage (Viarengo, 1989; Winston et al., 1996; Ringwood et al., 2002), while the frequency of micronuclei occurrence is an effective measure of DNA damage (Burgeot et al., 1996; Bolognesi et al., 2004).

Tellina deltoidalis is a sediment dwelling bivalve which is widely distributed in coastal estuaries around Australia where it lives buried in the sediments at a depth several times the shell length, of between 15 - 25 mm, and extends its siphons to the sediment surface to feed (Beesley et al., 1998). It satisfies most of the basic requirements to be an effective biomonitor being hardy, representative of the area of interest and an accumulator of bioavailable metals (Phillips, 1990; Phillips and Rainbow, 1994). The suitability of *T. deltoidalis* for use in whole sediment toxicity tests has been investigated by King et al. (2004; 2005; 2010) who found they were tolerant of a wide range of sediment types and salinities and easy to handle in a laboratory setting, while being sensitive to metal contamination. A protocol for the use of *T. deltoidalis* in whole-sediment acute toxicity tests has been included in the Australian Handbook for Sediment Quality Assessment (Simpson et al., 2005).

The purpose of this study was to examine the exposure - dose - response relationship to selenium in *T. deltoidalis* using 28 day sediment bioaccumulation tests (USEPA, 2000; ASTM-E1688-10, 2010) to develop useful biomarkers of effect, and further evaluate their potential for sediment toxicity testing in Australia using sublethal endpoints. There are no Australian sediment quality guideline concentrations for selenium so the exposure concentrations, 5 and 20 μg/g, chosen where based on those previously measured in contaminated Australian estuarine sediments (Peters et al., 1999a; Roach, 2005). Internal selenium exposure was measured in whole tissues, and subcellular tissue fractionation used to determine the active and detoxified selenium. Biomarker measurements of oxidative stress included total antioxidant scavenging capacity of cells, total glutathione concentrations, the ratio of reduced to oxidised glutathione, glutathione peroxidise and the extent of lipid peroxidation. Cellular damage was assessed using a lysosomal destabilisation assay and DNA damage through the presence of micronuclei. Measurement of enzymatic biomarkers in the glutathione cycle along with the cellular and genotoxic biomarkers of lysosomal membrane integrity and micronuclei occurrence provides a weight of evidence approach for

selenium toxicity at the individual organism level which may indicate the potential for population level effects.

2 Materials and Methods

2.1 Organism and sediment collection

Sediments were collected from a NSW Department of Environmental and Climate Change reference site in Durras Lake NSW, and stored at 4^{o}C until use. *Tellina. deltoidalis* of 15 – 20 mm in size were collected from Durras Lake and Lake Tabourie, NSW in July 2005 and January 2006 and placed in coolers with sediment and water from the collection sites for transportation. Organisms were maintained for a maximum of two weeks at 22^{o}C in uncontaminated sediments, depth 15 cm, in glass aquaria with filtration and aeration to allow acclimation before experimentation. Overlying water used in aquaria was collected from coastal waters near Murramurrang National Park, NSW and adjusted from 35‰ to 28‰ with deionised water to match the salinity of the estuarine water from which organisms were collected.

2.2 Sediment selenium spiking

Sediments were sieved through a 2 mm stainless steel sieve to remove large pieces of organic matter and organisms prior to the addition of selenium. Sub samples of the collected sediments were measured for moisture content and grain size. To ensure the sediment matrix was suitable for organism burrowing and feeding, sediment was mixed with clean beach sand so that the 63 µm fraction was not greater than 20% mass/mass. To ensure added selenium was rapidly adsorbed and strongly bound to the sediment particles a method developed by (Simpson et al., 2004) for producing metal spiked marine sediments, was followed. Wet sediment was added to mixing containers. Na_2SeO_3, (AR grade Sigma-Aldrich) was added to concentrations of 5 and 20 mg/kg dry mass of sediment. All containers were topped up with clean deoxygenated sea water and the final mixture was completely deoxygenated by bubbling with nitrogen for 2 hours. Head spaces of containers were filled with nitrogen prior to sealing. Any pH adjustments were made immediately after the addition of the selenium using 1M NaOH, (AR grade BDH), prepared in seawater, checked weekly and maintained at 7 - 8.2. Sediments were mixed on a Cell-production Roller Apparatus (Belco, USA) for several hours each day. Sediments were maintained at room temperature 22 - 25^{o}C. The time required for equilibration of added metals will be affected by the sediment properties, equilibration pH and the concentration and properties of the metal (Simpson et al., 2004).

To determine when the added selenium was completely bound to sediment particles, pore waters were collected and acidified to 1% v/v with nitric acid (AristaR, BDH, Australia) and selenium was measured using an ELAN® 6000 ICP-MS (PerkinElmer SCIEX, USA). Once pore water selenium concentrations had fallen below instrument detection limits 0.001 µg/l the sediment was ready for use. Time to full absorption was 4 to 6 weeks. Unspiked sediments were treated in the same way and used for control treatments. Sediment selenium concentrations were measured by ICP-MS after digestion of 0.2 g of lyophilised sediment in 3 ml of nitric acid (AristaR, BDH, Australia) in polyethylene 50 ml centrifuge tubes for 60 minutes at 115°C (Maher et al., 2003). Selenium in NRCC Certified Reference Materials, BCSS-1 marine sediment measured along with samples was 0.41 ± 0.1 µg/g (n = 10) and in agreement with certified values 0.43 ± 0.06 µg/g. Sediment selenium concentrations were measured prior to and at the end of the 28 day exposure period. Pre exposure concentrations were < 0.001, 5.00 ± 0.05 and 20 ± 1 µg/g and post exposure were < 0.001, 5.00 ± 0.15 and 19 ± 2 µg/g.

2.3 Microcosm Experiment Design

Procedures for conducting the exposures were adapted from the test method for conducting 28 day sediment bioaccumulation tests (Ingersoll et al., 2000). Spiked and control sediments (500 g wet mass) were placed in each of three replicate 770 ml polypropylene containers (Chanrol # 01C30, Australia) per treatment. The containers were filled with fresh seawater adjusted to a salinity of 28‰. Containers were placed in random order on a tray in an incubator set at 22°C with a day / night light cycle of 14 / 10 hours to reflect spring / summer conditions. Aeration was introduced and the treatments were left for 24 hours to allow them settle and the temperature to equilibrate. Fifteen *T. deltoidalis* were then introduced to each treatment container. Organisms were not given supplementary food and surface water was changed weekly during the 28 day exposure period. Aquaria were continually aerated using an air pump with valves on each line and fine tubing to each container to regulate air flow so oxygen saturation ≈ 100% were maintained in overlying water of each aquarium but sediments were not agitated. Due to the natural buffering capacity of sea water and associated sediments, pH remained relatively constant at 7.8-8.0 in all aquaria throughout the 28 days of exposure. This is similar to results of other studies of this type (King et al., 2006; Strom et al., 2011). Total tissue selenium bioaccumulation was measured at intervals of 3, 7, 14, 21 and 28 days. A day 0 measurement was made using organisms from the acclimation tanks to

give the background selenium concentration. All organisms were placed in fresh seawater at salinity 28‰ with no sediment for 24 hours (King et al., 2004; Simpson et al., 2005; Atkinson et al., 2007; King et al., 2010) to allow depuration of ingested sediment particles, prior to selenium analysis. All assays were done on whole tissues of individual organisms.

2.4 Selenium Measurements

2.4.1 Total selenium

Lyophilised ground tissue ≈ 0.1 g was digested in 1 ml of nitric acid (AristaR BDH, Australia) in polytetra-fluroacetate digestion vessels, in a 630 watt microwave oven (CEM MDS-2000, USA) for 2 min at 630 W, 2 min 0 W, and 45 min at 315 W (Baldwin et al., 1994). Prior to analysis samples were diluted with deionised water to 1% v/v HNO_3, and an ICP-MS mixed 7-element internal standard (EM Science) was added to monitor for variations due to instrument drift and/or matrix effects. Selenium was measured using an ELAN® 6000 ICP-MS (PerkinElmer, SCIEX) following the method of Maher et al. (2001). NRCC Certified Reference Material, NIST 1566a oyster tissue and acid blanks were routinely digested and diluted in the same way as the samples and analysed along with them to verify accuracy and precision of selenium analysis. The measured CRM mean selenium value; 2.1 ± 0.3 µg/g (n = 50) was not significantly different from the certified value 2.21 ± 0.24 µg/g.

2.4.2 Subcellular selenium

The subcellular tissue selenium distribution was examined in tissues of day 28 exposed organisms using a procedure adapted from Sokolova et al. (2005) and Wallace et al. (2003). The dissected tissues were placed in polypropylene vials, snap frozen in liquid nitrogen and stored at -80^{o}C until processed. The tissue was thawed and minced on ice with a blade. A sub sample, ≈ 0.1 g wet wt., was taken for total tissue selenium analysis. The remainder, ≈ 0.5 g wet wt., was homogenised in Ca^{2+} / Mg^{2+} free saline buffer pH 7.35 on ice using an IKA® Labortechnick Ultra-turrax-T25 homogeniser equipped with an S25-UT dispersing tool at 9,500 $rpmin^{-1}$ (Janke & Kunkel, Germany). Homogenised tissue was subjected to differential centrifugation and tissue digestion procedures according to the protocol outlined in Taylor (2009), using an Eppendorf 5804R centrifuge and a Himac CP90WX preparative ultracentrifuge (Hitachi, Japan). The mitochondria, lysosomes-microsomes and heat sensitive protein pellets were grouped as biologically active selenium fractions while the granule and heat stable metallothionein like proteins were grouped as biologically detoxified selenium

fractions (Taylor and Maher, 2013). The supernatant from the granule pellet isolation contained the nuclei-cellular debris. To determine the mitochondrial and lysosomal content of the fractions obtained the concentration of enzymes specific for these organelles, cytochrome *c* oxidase and acid phosphatase, respectively, were measured in each of the total tissue, mitochondrial and lysosome-microsome pellets using commercial colorimetric assays (CYTOC-OX1 Sigma-Aldrich, USA and CS0740 Sigma-Aldrich, USA, respectively). This showed that the mitochondrial fraction was enriched with mitochondria and in the lysosome-microsome fraction there was some enrichment of lysosomes compared to the mitochondrial fraction (Supplementary Figure 1). Fractions were acidified to 10% v/v with nitric acid (AristaR BDH, Australia) and placed in a water bath at 80^{o}C for 4 hours. NIST CRM 1566a oyster tissue, buffer and acid blanks were digested and diluted in the same way as the samples and analysed along with them. Analysis of selenium was as previously described above. The measured CRM selenium value 2.25 ± 0.3 µg/g (n = 5) were in good agreement with certified value 2.21 ± 0.24 µg/g.

2.5 Biomarker Measurements

2.5.1 Total antioxidant capacity and lipid peroxidation

Tissues were homogenised on ice in a 5 mM potassium phosphate buffer containing 0.9% (w/v) sodium chloride and 0.1% (w/v) glucose, pH 7.4 (1:5 w/v) using a motorised microcentrifuge pellet pestle, sonicated on ice for 15 seconds at 40 V (VibraCell™ Sonics Materials, USA) and centrifuged, in a 5804R centrifuge (Eppendorf, Germany), at 10,000 x g for 15 minutes at 4^{o}C (Cayman, 2011). The supernatant was stored at -80^{o}C until analysis. Total antioxidant capacity was measured using an assay based on the ability of the tissue lysate antioxidant system to inhibit the oxidation of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS $^{\bullet+}$ by metmyoglobin in the presence of hydrogen peroxide. This was compared with the antioxidant capacity of a standard, Trolox (Cayman, 2011). Samples were pipetted into a 96 well plate with metmyoglobin and ABTS. The reactions were initiated with a 441 µl solution of hydrogen peroxide. The plate was shaken for 5 minutes at 25^{o}C and the amount of ABTS$^{\bullet+}$ produced was measured by the suppression of absorbance at 750 nm on a BioRad Benchmark Plus microplate spectrophotometer. This is proportional to the final total antioxidant capacity concentration, expressed in millimolar Trolox equivalents. The Thiobarbituric Acid Reactive Substances (TBARS) assay was used to measure lipid peroxidation by measuring the malondialdehyde (MDA) concentration in

each tissue lysate. The end product of lipid peroxidation, MDA, forms a 1:2 adduct with TBARS and produces a colour reaction that can be read spectrophotometrically at 532 nm and compared to an MDA standard curve (ZepoMetrix, 2011). The samples were incubated in a solution of sodium dodecyl sulphate, thiobarbituric acid and sodium hydroxide dissolved in acetic acid at 95^{o}C for 60 minutes. After cooling on ice and centrifuging at 3000 rpm for 10 minutes at room temperature, the colour reaction was measured, on a BioRad Benchmark Plus microplate spectrophotometer at 532 nm.

2.5.2 Reduced:oxidised glutathione ratio and glutathione peroxidase

Tissue lysates were produced by homogenisation on ice in a 50 mM Tris-HCl buffer containing 5 mM EDTA and 1 mM DTT, pH 7.5 (1:5 w/v) using the technique outlined above. A thiol scavenging agent 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate in HCl (Calbiochem®, Merck, Germany) was added to GSSG tissue homogenates to remove GSH, prior to the addition of buffer and production of the final supernatant. The remaining GSSG is then reduced to GSH and determined by the reaction with Ellman's reagent (Calbiochem, 2004). Supernatants were stored at -80^{o}C until analysis of reduced glutathione (GSH), glutathione peroxidise (GPx) and protein (Calbiochem, 2004). The ratio of reduced to oxidised glutathione (GSH:GSSG) was measured using an enzymatic method based on one developed by (Tietze, 1969). The method uses Ellman's reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) which reacts with GSH to form a colour which is detected at 412 nm (Calbiochem®, Merck, Germany). The samples were acidified by the addition of a 5% solution of metaphosphoric acid, vortexed for 15 seconds and centrifuged at 1000 x g for 10 minutes at room temperature. The metaphosphoric acid extracts were diluted with a sodium phosphate buffer and mixed at room temperature in 1 ml cuvettes with DTNB and glutathione reductase enzyme at (1:1:1 v/v/v). The reaction was initiated with ß - nicotinamide adenine dinucleotide phosphate (NADPH) and absorbance read at 412 nm for 3 minutes at intervals of 15 seconds on a Unicam Helios Gamma UV-Vis spectrophotometer (Spectronic, UK). Absorbance rates were calculated and GSH and GSSG concentrations calculated using a 6 point GSH calibration curve. A GSSG buffer blank was run for interference correction. Glutathione peroxidise activity (GPx) was measured using a coupled reaction with glutathione reductase (GR) (Cayman Chemicals, USA). The oxidation of NADPH to $NADP^{+}$ is accompanied by a decrease in absorbance at 340 nm. Under conditions where GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample. Assay buffer 50 mM Tris-HCl, pH 7.6, 5 mM EDTA was added to sample wells of a

flat bottomed 96 well plate with a co-substrate mixture NADPH, glutathione and GR (2:1 v/v). Samples were added to each well and the reaction was initiated by the addition of cumene hydroperoxide. The plate was shaken briefly and the decrease in absorbance read at 340 nm for 5 minutes at intervals of 30 seconds at 25^{o}C on a BioRad Benchmark Plus microplate spectrophotometer. Rates were calculated and samples were compared with a bovine erythrocyte GPx positive control. Buffer blanks run with the samples were used to correct for interferences and GPx activity was calculated using the NADPH extinction coefficient, adjusted for the pathlength of the solution, of 0.00373 μM^{-1}. One unit is defined as the amount of enzyme that will cause oxidation of 1.0 nmol of NADPH to $NADP^{+}$ per minute at 25^{o}C.

2.5.3 Protein

All tissue lysates used for enzymatic assays were analysed for protein concentration and enzyme concentration / activity is expressed as mg^{-1} of protein in the sample. The FluoroProfile™ (Sigma #FP0010, Sigma-Aldrich, USA) protein assay used is a fluorescent assay based on Epiccoconone, a biodegradable natural product. The fluorescence intensity was read at 485 nm excitation and 620 nm emission, on a Luminoskan Ascent Fluorescence Plate Reader (Thermo Electrical Corp., USA). Bovine serum (BSA) calibration curve standards used were made up in sample buffer.

2.6 Cellular and Genotoxic Biomarkers

2.6.1 Lysosomal Stability

Lysosomal stability was assessed using a method developed by (Ringwood et al., 2003) for oysters. The assay uses neutral red (NR) dye retention to assess the integrity of the lysosomal membrane. Cells incubated in neutral red accumulate the lipophilic dye in the lysosomes. Healthy cells retain the dye in the lysosomes whereas in cells with damaged lysosomal membranes it leaks out into the cytoplasm. Minced tissue was shaken in CMFS buffer pH 7.35 salinity 30‰ on a reciprocating shaker at 100 rpm for 20 minutes. Trypsin (T4799 Sigma, USA), 325 µl at 1 mg/ml in CMFS buffer, was added and samples shaken for a further 20 minutes. Cells were then collected by centrifuging samples through a 20 µm screen at 250 - 500 *x* g at 15^{o}C for 5 - 15 minutes. Cells were incubated in neutral red (Sigma, USA), 0.04 mg/ml in CMFS for 1 hour and one hundred cells per slide were counted using a light microscope with 40x lens and scored as stable or unstable, based on dye retained in the lysosomes or present in the cytosol, respectively. Two slides per sample were counted.

2.6.2 Micronuclei Frequency

The micronuclei assay used was based on a technique developed on the mussel *Mytilus galloprovincialis* (Gorbi et al., 2008). The assay uses DAPI (4',6-diamidine-2'-phenylindole dihydrochloride), a fluorescent dye specific for nucleic material, to stain the nuclei. Micronuclei are defined as small round structures less than one third the diameter and in the same optical plan as the main nucleus, with a boundary distinct from the nuclear boundary. Tissue preparation for the collection of cells was the same as that used for the neutral red retention assay. The rinsed cells were fixed in Carnoy's solution (methanol:glacial acetic acid 3:1) and stored at 4^{o}C until counted. A drop of the fixed cell suspension was placed on a slide and air dried. A drop of the DAPI (# 32670 Sigma, USA) working solution was added to each slide and a cover-slip added. Slides were incubated in the dark for 5 minutes and observed under an inverted epifluorescent microscope (Nikon, Eclipse TE 300, Japan) with the appropriate filter for DAPI, excitation wavelength 350 nm magnification 40x. Two slides per sample were counted with 1000 cells per slide scored as micronuclei present or absent.

2.7 Statistical analyses

A Mixed Linear Model analysis of variance (ANOVA) (SPSS *v* 14.0) was used to simultaneously analyse the effects of time (day) and treatment (selenium exposure concentration) on organism tissue selenium accumulation. A Mixed Linear Model ANOVA was used to analyse the effects of treatment (selenium exposure concentration) on the effect measurement variables antioxidant capacity, total glutathione, GSH:GSSG ratio, glutathione peroxidase, lipid peroxidation, lysosomal stability and micronuclei frequency. (Supplementary Tables 1 – 3). Regressions of sediment selenium and mean tissue selenium concentrations and means of effects variables antioxidant capacity, lipid peroxidation, lysosomal stability and micronuclei frequency were calculated using EXCEL™ *v* 2003 (Supplementary Table 4).

3 Results

3.1 Selenium Accumulation

Selenium tissue concentrations in organisms from both selenium treatments differed significantly from the unexposed and control organisms ($p \leq 0.0005$) but not from each other (Figure 1; Supplementary Tables 1 & 2). The highest selenium concentrations for both treatments were at day 21 with a slight but not significant decrease to day 28 (Figure 1). At day 28, selenium tissue concentrations in the both treatments were higher than the selenium

spiked sediment with the 5 µg/g treatment organisms having 5 times and the 20 µg/g 1.5 times the sediment selenium concentration (Figure 1). After 28 days of exposure there was a significant positive relationship ($r = 0.46$; $p \leq 0.0001$; $n = 41$) between sediment and organism tissue selenium concentrations (Supplementary Table 4).

3.1.1 Subcellular Tissue Selenium Distribution

Approximately 75% the total selenium from the organisms exposed to sediment concentrations of 5 and 20 µg/g selenium was recovered in the fractions with 82% recovered from the controls (Table 1). Of the recovered selenium, up to 60% was in the nuclei-cell debris fraction in the selenium treatments (Table 1). The biologically active selenium burden was 1.8 and 2.8 times respectively, greater in the 5 and 20 µg/g selenium exposed organisms than the controls (Table 1). The mitochondrial fraction contained the highest percentage of biologically active selenium in the 5 and 20 µg/g selenium exposed organisms, followed by the heat sensitive protein fraction, with only a small percentage in the lysosome-microsome fraction (Figure 2; Table 2). The control organisms also had the lowest percentage of biologically active selenium in the lysosome-microsome fraction while the heat sensitive protein fraction had the highest percentage with slightly less in the mitochondria (Figure 2; Table 2). The majority of biologically detoxified selenium was in the granule fraction accounting for 97% in the control organisms and 66 and 77%, respectively, in the 5 and 20µg/g selenium exposed organisms (Figure 2; Table 2). Selenium exposed organisms had a higher concentration of the mitochondrial enzyme cytochrome *c* oxidase than the control organisms (Supplementary Figure 1). At the highest selenium exposure the concentration of the lysosomal enzyme acid phosphatase was increased in both whole tissue and in the lysosome-microsome fraction (Supplementary Figure 1).

3.2 Biomarkers

The total antioxidant capacity (TAOC) of the selenium exposed organisms was significantly reduced ($p \leq 0.01$; Supplementary Table 3a) compared to that of unexposed organisms, however, the TAOC of each of the selenium treatments were not significantly different to each other (Figure 3A; Supplementary Table 3b). Compared to control organisms the glutathione peroxidase (GPx) activity and total glutathione concentrations were enhanced in the selenium treatments (Figure 3B) but the difference was not significant ($p > 0.05$; Supplementary Table 3b). The ratio of reduced and oxidised glutathione was significantly reduced in selenium exposed organisms compared to that of unexposed organisms ($p \leq 0.01$;

Supplementary Table 3a); however, the ratios of each of the selenium treatments were not significantly different to each other (Figure 3B). Thiobarbituric acid reactive substances were significantly higher in selenium exposed organisms than in unexposed organisms ($p \leq 0.05$; Supplementary Table 3a). The organisms from the selenium exposures both had significantly higher lipid peroxidation (TBARS) than the controls but not from each other (Figure 4A; Supplementary Table 3b). Selenium exposed organisms had significantly more unstable lysosomes and a higher frequency of micronuclei than the control organisms ($p \leq 0.001$; Supplementary Tables 3a). The 20 µg/g selenium exposed organisms had significantly more unstable lysosomes and a higher frequency of micronuclei than both the control and 5 µg/g selenium exposed organisms ($p \leq 0.001$; Supplementary Tables 3b; Figures 5B & C). Regression analysis showed that when selenium exposure reduced the TAOC within cells this corresponded with an increase in the effects measures of TBARS ($r = 0.37$; $p \leq 0.0001$; $n = 36$), lysosomal destabilisation ($r = 0.41$; $p \leq 0.01$; $n = 18$) and micronuclei frequency ($r = 0.51$; $p \leq 0.001$; $n = 18$) (Supplementary Table 4). As TBARS increased there was a corresponding increase in lysosomal destabilisation ($r = 0.32$; $p \leq 0.01$; $n = 18$) and the frequency of micronuclei ($r = 0.35$; $p \leq 0.01$; $n = 18$) (Supplementary Table 4).

4 Discussion

4.1 Selenium Accumulation and Subcellular Distribution

4.1.1 Whole tissue

The variation in tissue selenium concentrations over time in the control organisms was in the order of a few micrograms per gram (Figure 1). As selenium is an essential element some basal concentration is expected (Hamilton, 2004), so this would be indicative of natural variation. Selenium accumulation was rapid during the first three days of exposure in organisms from both selenium treatments, with the 5 µg/g selenium exposed organisms accumulating twice the exposure concentration and the 20 µg/g equalling it in this time (Figure 1). A similar equilibrium tissue concentration for organisms from both treatments appears to have been reached after four weeks (Figure 1). Peters et al. (1999b) found native *T. deltoidalis* exposed to sediment selenium concentrations of 3.4 µg/g in Lake Macquarie NSW accumulated tissue concentrations of 32 µg/g which is a considerably higher exposure to tissue selenium ratio than observed for the *T. deltoidalis* in this experiment. Selenite is taken up rapidly by the aquatic microflora and fauna that is consumed directly by deposit feeding bivalves from the sediment surface or as part of the detritus. In addition selenite

adsorbed to sediment particles may be ingested (Fan et al., 2002; Hamilton, 2004). *T. deltoidalis* in our experiments may have had a change in selenium exposure route from an initial direct absorption from ingested sediment particles to a later additional dietary exposure as microfauna and flora, present in the natural sediments and water used, assimilated selenium and were consumed. The major route of selenium uptake in aquatic systems is via food rather than as the free ions in solution (Luoma and Rainbow, 2008), therefore the final greater than ambient selenium tissue concentrations observed in this experiment after 21 days exposure may be related to the generation of a source of dietary selenium.

4.1.2 Subcellular selenium distribution

A large proportion of the selenium recovered in the subcellular fractions was in the nuclei-cellular debris fraction, increasing from 36% in the controls to 56 and 60%, respectively, in the 5 and 20 µg/g exposed organisms (Table 1; Figure 2). Selenite is bound to plasma proteins for transport to tissues. It has been suggested that selenite is taken up by haemolymph, reduced to selenide, released into the plasma and rapidly bound by plasma proteins (Ewan, 1989). The majority of accumulated selenate and selenomethionine occurs in the plasma (Ewan, 1989). Selenomethionine has been shown to bind to glutathione peroxidase extracellularly as well as intracellularly (Burk, 1991). Mycelia of the fungus *Pleurotus ostreatus* enriched with selenium had 56% of accumulated selenium associated with the cell wall (Hortensia et al., 2006). A combination of protein bound selenium associated with plasma and selenium bound directly to cell walls would account for the high proportion of selenium associated with this fraction and therefore it would be comprised of both biologically active and detoxified selenium. Of the remaining selenium recovered in the fractions, the control organisms had 11% and the exposed organisms 20% in the detoxified selenium fractions (Table 1; Figure 2), but the distribution within this portion differed. The control organisms had most selenium in the granule fraction while the 5 and 20 µg/g exposed organisms had 66 and 77%, respectively, in the granules with the remainder in the metallothionein like proteins (Table 2). Selenium associated with metallothionein like proteins has not previously been reported in aquatic organisms, although there is evidence from mammalian studies that suggests selenite exposure induces metallothionein production (Iwai et al., 1988; Chen and Whanger, 1994). The majority of selenium not associated with selenoproteins of the glutathione peroxidase family has been found bound to selenoamino acids and other low molecular weight selenium compounds analogous to metallothioneins and it is presumed that these act as storage and transport proteins and intermediaries in the

synthesis of selenoproteins (Akesson and Srikumar, 1994). The metallothionein like protein fraction may therefore represent a pool of detoxified selenium. Like metallothioneins, selenium associated with granules has not previously been described. The operational fraction defined as granules in this procedure has been examined in fractions obtained from cadmium exposed oligochaetes by Wallace et al. (1998), with a compound microscope, and shown to contain numerous metal rich granules of varying sizes. The fraction obtained in the present study using the same technique as described by Wallace et al. (1998) was not examined visually for granules so it can only be assumed that the fraction contained detoxified selenium rich concretions. Using a similar fractionation procedure Zhang and Wang (2006) found 40% and 60% of accumulated selenium in crustaceans and bivalves, respectively, was associated with the granule fractions, while Dubois and Hare (2009) obtained only 1 - 2% of selenium in the granule fractions of the oligochaete *Tubifex tubifex* and the insect *Chironomus riparius*. George, (1983) showed that granules of cadmium exposed *Mytilus edulis* contained high concentrations of protein, calcium and sulphur. Selenium is known to substitute for sulphur in proteins as it has similarities with the chemistry of sulphur (Ewan, 1989). The presence of selenium in a protein is always related to the presence of sulphur, the selenium atom is either incorporated in the place of a sulphur atom in a sulphur amino acid, or it is attached to the sulphur atoms of cysteine residues (Ganther, 1974). It is possible that selenium is incorporated into granule like structures via a similar process to that postulated for cadmium by George (1983), due to an increase in lysosomal protein degradation, following enzyme inactivation by intracellular selenium, causing an increase in intracellular protein turnover. Alternatively the granule fraction may represent selenium associated with incompletely digested tissue and cell debris in the NaOH digestion step of the fractionation procedure (Taylor and Maher, 2013). The fractionation procedure used by Zhang and Wang (2006) which found 40 and 60% of selenium in crustaceans and bivalves associated with the granule fraction used a shorter NaOH digestion step, 10 minutes rather than the 60 minutes used in this study, so incomplete digestion of the tissue and cell debris fraction in their study is also a possibility. If this is the case then it is still a reasonable assumption that a fair proportion of this fraction represents detoxified selenium. The increased percentage of selenium associated with the detoxified selenium fractions (Figure 2) demonstrates that selenium detoxification processes are operating. The concentration of the mitochondrial enzyme cytochrome *c* oxidase was increased in the total homogenate and mitochondrial fractions of the selenium exposed organisms (Supplementary Figure 1) indicating an increased response in this organelle to selenium

accumulation. This is in agreement with the organelle selenium distribution results (Table 2; Figure 2) which show a 3 and 4 fold increase, respectively, in mitochondrial selenium in the 5 and 20 µg/g exposed organisms. As selenium is an essential component of the glutathione peroxidase enzyme it is expected that it will be present in the mitochondria where oxygen reduction and cellular energy production occurs, however, selenium toxicity can arise at concentrations only slightly greater than those that are required (Palace et al., 2004). The percentage of selenium in the heat sensitive protein fraction was slightly lower than that of the mitochondria in the selenium exposed organisms and higher in the controls (Table 2). This fraction contains enzymes, high and low molecular weight proteins and other target molecules which are sensitive to metals (Wallace et al., 2003). The increased binding of selenium in this fraction is not unexpected as selenium is largely associated with protein complexes (Ganther, 1974). The activity of the lysosomal enzyme acid phosphatase was only increased in the 20 µg/g exposure while the 5 µg/g organisms remained the same as the control organisms (Supplementary Figure 1). The percentage of biologically active selenium in the lysosomal-microsomal fraction of the selenium exposed organisms was around half that of the controls (Table 2) but the selenium concentration was 1.1 and 1.6 times higher in the 5 and 20 µg/g treatments, respectively, than the controls. The microsomal component of the cell includes fragmented endoplasmic reticulum, which is generally responsible for protein synthesis and transport, selenium in this fraction may be associated with microsomes rather than lysosomes which could be indicative of essential activity but equally could have implications for toxicity (Bonneris et al., 2005).

4.2 Enzymatic Biomarkers – Oxidative Enzymes

Selenium is an essential element involved in the reduction of peroxide in the glutathione cycle (Micallef and Tyler, 1987; Hodson, 1988; Hoffman, 2002). Total antioxidant capacity was significantly reduced in the selenium exposed *T. deltoidalis* compared to the control organisms but there was no difference in antioxidant capacity between selenium treatments (Figure 3A). The similarity in the antioxidant response between selenium treatments may be explained by the similarity in the final selenium tissue concentrations between the two treatments (Figure 1). Changes in activity and concentration of enzymes within the glutathione cycle indicate an imbalance in the intracellular glutathione redox status. The activity of the glutathione peroxidase (GPx) enzyme was enhanced in the *T. deltoidalis* from both selenium exposures although not significantly compared to control organisms (Figure 3B). The increase in GPx activity may have increased the rate of oxidation of GSH as seen in

the increased GSH+2GSSG concentration and the significantly reduced GSH:GSSG ratio (Figure 3B; Supplementary Table 3b). The increase in GSSG may also be a result of the direct reaction of selenite with GSH, which has been shown in the trout *Oncorhynchus mykiss* to produce increased reactive oxygen species, a sharply decreased GSH/GSSG ratio and increased membrane lipid peroxidation (Misra and Niyogi, 2009). Studies in mallard ducks showed that increased dietary and subsequent selenium tissue concentrations resulted in increases in plasma and hepatic GPx activity and GSH concentrations, followed by a dose-dependent decrease in the ratio of hepatic GSH to GSSG concentrations which ultimately led to increased hepatic lipid peroxidation (Hoffman, 2002). Excess GSSG can react with protein sulfhydryls, contributing to the total thiol and protein bound thiol depletions, by the formation of mixed glutathione:protein disulphides. Formation of mixed disulphides may be part of a significant mechanism in regulating metabolic activity as well as the integrity of the cell membranes in response to oxidative stress (Hoffman, 2002). An examination of selenomethionine metabolism in embryos of the trout *Oncorhynchus mykiss* showed oxidative stress, which appeared to be generated by methioninase enzyme activity, liberating methylselenol from L-Selenomethionine (Palace et al., 2004). The methylselenol is able to undergo redox cycling in the presence of glutathione producing superoxide and likely accounts for oxidative stress measured in aquatic organisms environmentally exposed to excess selenomethionine (Palace et al., 2004). Although the sediment in this study was spiked with sodium selenite, which is readily bioaccumulated by animals and bound to proteins following assimilation into cells, animals do not have the capacity to transform it into selenomethionine (Suzuki and Ogra, 2002; Suzuki et al., 2006). Marine algae and bacteria, however, are known to convert selenite mainly into selenomethionine (Fan et al., 2002; Orr et al., 2006) and this secondary pathway of dietary derived selenium may have resulted in selenomethionine exposure for *T. deltoidalis* during the course of the experiment.

4.3 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) are a measure of lipid peroxidation, a widely recognised consequence of excess oxyradical production which destabilises cell membranes leading to loss of lysosomal integrity and the leaking of the lysosomal contents into the cytoplasm (Winston, 1991; Winston and Di Giulio, 1991). The concentration of TBARS increased significantly in *T. deltoidalis* from both selenium treatments compared to the control organisms (Figure 4A). Increased hepatic lipid peroxidation related to effects of accumulated selenium on glutathione metabolism have been measured in a number of wild

aquatic birds, including their hatchlings and eggs (Hoffman, 2002). The TBARS concentration of the selenium exposed organisms was highly negatively correlated with the total antioxidant capacity, indicating that the increased tissue selenium resulted in a reduction in the capacity to reduce reactive oxygen species. It is likely that their subsequent increase directly influenced the build-up of lipid peroxidation by-products.

4.4 Cellular Biomarker – Lysosomal Stability

Metal accumulation in the lysosomes can induce lipid peroxidation through redox cycling or by direct reaction with cellular molecules to generate reactive oxygen species (Ercal et al., 2001). This can destabilise the lysosomal membrane causing the contents to leak out into the cytosol thereby reducing the cells capacity to remove waste which will ultimately lead to cell death (Viarengo et al., 1987). Similar to *T. deltoidalis* exposed to cadmium and lead (Taylor and Maher, 2013; 2014), the selenium exposed *T. deltoidalis* had significantly higher lysosomal destabilisation than the control organisms (Figure 4B). The 5 µg/g selenium exposed *T. deltoidalis* were in the 'concern range' with 30% destabilised lysosomes, while the 20 µg/g selenium exposed organisms would be classed as 'stressed' with 68% lysosomal destabilisation based on the Ringwood et al. (2003) criteria. The biologically active selenium burden of the 20 µg/g selenium exposed organisms was ≈ 1.6 times that of the 5 µg/g selenium exposed organisms and this may account for the significantly higher lysosomal membrane damage. The lysosomal fraction of the selenium exposed organisms did not have a marked selenium burden increase, the majority of active selenium was associated with the mitochondrial and heat sensitive protein fractions (Table 2). Selenium binding to molecules present in the heat sensitive proteins of the cytosol may contribute to the total thiol and protein bound thiol depletions, which may be part of a significant mechanism in regulating metabolic activity as well as the integrity of the cell membranes in response to oxidative stress (Hoffman, 2002).

4.5 Genotoxic Biomarker – Micronuclei Frequency

The micronuclei test is a sensitive test to detect genomic damage due to both clastogenic effects and alterations to the mitotic spindle (Migliore et al., 1987). It has been used in bivalves to examine the genotoxicity of a range of chemicals (Scarpato et al., 1990; Williams and Metcalfe, 1992; Burgeot et al., 1996; Bolognesi et al., 2004). The occurrence of micronuclei increased significantly with selenium exposure (Figure 4C). Induction of micronuclei in response to selenium bioaccumulation has not previously been investigated in bivalves, however, these results fit the pattern found for metal induced genotoxic damage as

increased frequency of micronuclei shown for *Mytilus galloprovincialis* (Dailianis et al., 2003; Bolognesi et al., 2004; Kalpaxis et al., 2004; Gorbi et al., 2008) and for *T. deltoidalis* exposed to cadmium and lead (Taylor and Maher, 2013; 2014). Increased micronuclei frequency in response to selenium exposure has been observed in fish erythrocyte cells (al Sabti, 1994) and mice bone marrow (Itoh and Shimada, 1996). The frequency of micronuclei in the selenium exposed *T. deltoidalis* corresponded with a decrease in antioxidant capacity and an increase in lipid peroxidation (Supplementary Table 4) indicating that an increase in ROS contributed to an increase in genotoxic damage, either through interaction of reactive oxygen intermediates and lipid peroxidation products with DNA or direct interaction of selenium with cellular macromolecules forming adducts, alkaline labile sites and strand breaks (Regoli et al., 2004).

5 Summary and Conclusions

This study has demonstrated a significant exposure – dose – response relationship for selenium in *T. deltoidalis*. Exposure to selenium contaminated sediments resulted in selenium bioaccumulation but not in proportion to the sediment selenium concentrations. Up to 60% of the accumulated selenium was in the nuclei-cellular debris fraction probably comprised of a combination of protein bound selenium associated with plasma and selenium bound directly to cell walls and therefore effectively removed from active sites within the cell. The percentage of selenium increased in the biologically detoxified fraction of selenium exposed organisms and was associated with both granules and metallothionein like proteins, which has not previously reported for marine bivalves. Selenium associated with low molecular weight proteins is likely to act as storage and transport and intermediaries in the synthesis of selenoproteins and therefore may represent a pool of detoxified selenium. The strong association of selenium with sulphur is a likely mechanism for the incorporation of selenium into granules as has been demonstrated for cadmium. Biologically active selenium burdens increased with selenium exposure and this led to impairment of the antioxidant system which may have initiated the observed increase in lipid peroxidation, lysosomal destabilisation and micronuclei frequency.

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Figure & Table Legends

Figure 1: Tissue selenium concentrations (µg/g dry mass) of *T. deltoidalis* exposed to selenium spiked sediments of 0 (control), Se 5 and 20µg/g dry mass. Mean ± SE, $n = 12$. Day 0 are unexposed organisms $n = 6$. Different letters indicate significant differences between means within treatments among collection day (Bonferroni test: $p < 0.05$).

Figure 2: Distribution (%) of selenium in each of the subcellular fractions of *T. deltoidalis* following 28 days exposure to selenium spiked sediments. Subcellular fractions are: Nuclei-cellular debris; granules; heat stable, metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes- microsomes (Lys & Mic); heat sensitive proteins (HSP). Stippled fractions (⁘) make up the biologically active selenium (BA), dashed fractions (▨) make up the biologically detoxified selenium (BD), $n = 2$.

Figure 3: Antioxidant enzyme biomarkers of *T. deltoidalis* after 28 days exposure to selenium spiked sediments of 0 (control), Se 5 and Se 20µg/g dry mass. Mean ± SE, $n = 12$. **3A**: TAOC (Total Antioxidant Capacity); **3B**: GPx (glutathione peroxidise); GSH+2GSSG (total glutathione); GSH/GSSG (ratio of reduced to oxidised glutathione). Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

Figure 4: Changes in oxidative damage biomarkers: **4A**: MDA (lipid peroxidation); **4B**: cellular (lysosomal destabilisation); and **4C**: genotoxic (micronuclei) of *T. deltoidalis* after 28 days exposure to selenium spiked sediments, 0 (control), Se 5 and Se 20µg/g dry mass. Mean ± SE $n = 12$. Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

Table 1: Total selenium concentrations (µg/g wet mass) in whole tissue and subcellular fractions with the percentage of total selenium recovered in all fractions of *T. deltoidalis* after 28 days exposure to selenium spiked sediments. Selenium subcellular concentrations (µg/g wet mass) and percentage distribution of total recovered selenium fractions are grouped as nuclei-cellular debris and biologically active and biologically detoxified selenium. Mean ± SE, $n = 2$.

Table 2: Mean percentage of selenium in the debris, biologically detoxified selenium (BD) and biologically active selenium (BA) with the percentage of selenium each of the fractions contributes to BD or BA of *T. deltoidalis* subcellular fractions after 28 days exposure to selenium spiked sediments. Mean ± SE, $n = 2$.

1019 **Table 1**:

	Sediment Treatments (µg/g)		
	control	Se 5	Se 20
Total Tissue Selenium (µg/g)	**0.42 ± 0.07**	**1.9 ± 0.5**	**3.4 ± 0.7**
Total Recovered Selenium (µg/g)	**0.34 ± 0.01**	**1.4 ± 0.5**	**2.5 ± 0.2**
Proportion of total recovered in fractions (%)	82 ± 12	75 ± 4	74 ± 10
Selenium Subcellular Distribution			
Nuclei - Cellular debris (µg/g)	**0.12 ± 0.004**	**0.78 ± 0.21**	**1.5 ± 0.2**
Nuclei - Cellular debris (%)	36 ± 1	56 ± 5	60 ± 4
Biologically Active Selenium (BA) (µg/g)	**0.18 ± 0.001**	**0.32 ± 0.1**	**0.51 ± 0.05**
Biologically Active Selenium (%)	53 ± 0.8	23 ± 1	20 ± 4
Biologically Detoxified Selenium (BD) (µg/g)	**0.04 ± 0.0003**	**0.29 ± 0.14**	**0.51 ± 0.08**
Biologically Detoxified Selenium (%)	11 ± 0.5	21 ± 4	20 ± 2

1020 Mean ± SD, $n = 2$

1021

1022 **Table 2**:

	Sediment Treatments (µg/g)		
	control	Se 5	Se 20
Nuclei - Cellular debris % of total	**36 ± 1**	**56** ± 5	**60 ± 4**
Biologically Detoxified Selenium % of total	**11 ± 0.5**	**21 ± 4**	**20 ± 2**
Selenium Rich Granules % of BD	97 ± 0.5	66 ± 5	77± 6
Heat Stable MT Like Proteins % of BD	3 ± 0.5	34 ± 4	23 ± 3
Biologically Active Selenium % of total	**53** ± 0.8	**23** ± 1	**20** ± 4
Mitochondria % of BA	34 ± 4	53 ± 1	48 ± 2
Lysosomes - Microsomes % of BA	22 ± 0.1	14 ± 2	12 ± 0.4
Heat Sensitive Proteins % of BA	44 ± 4	34 ± 1	40 ± 3

1023

1024 **Figure 1**

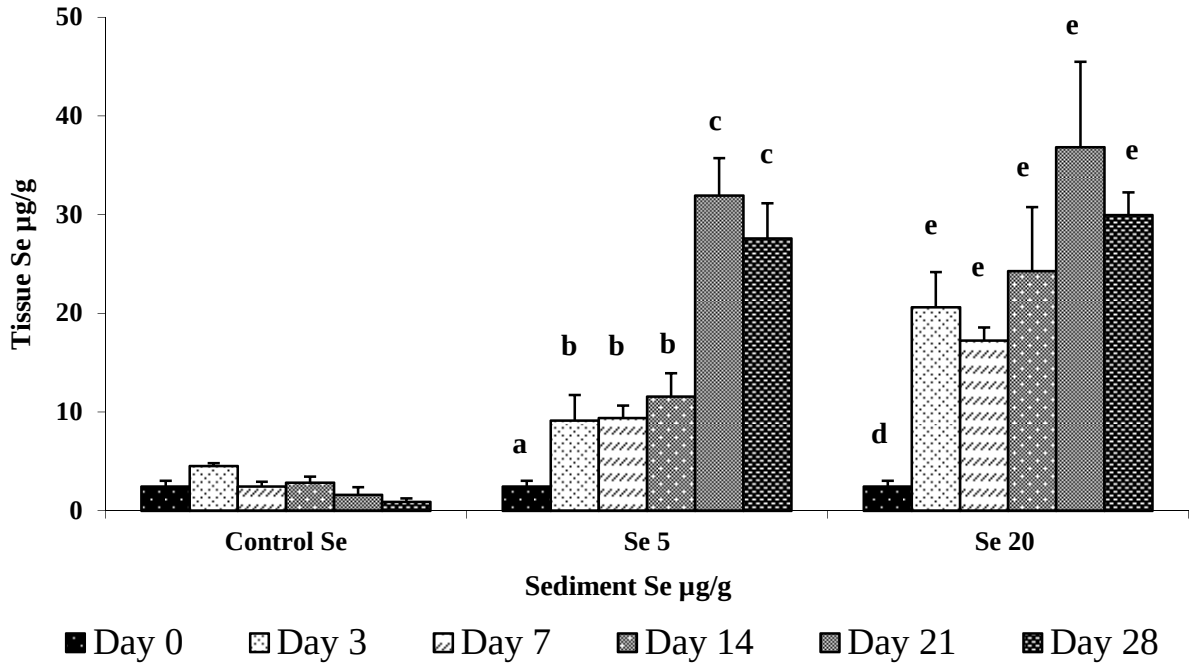

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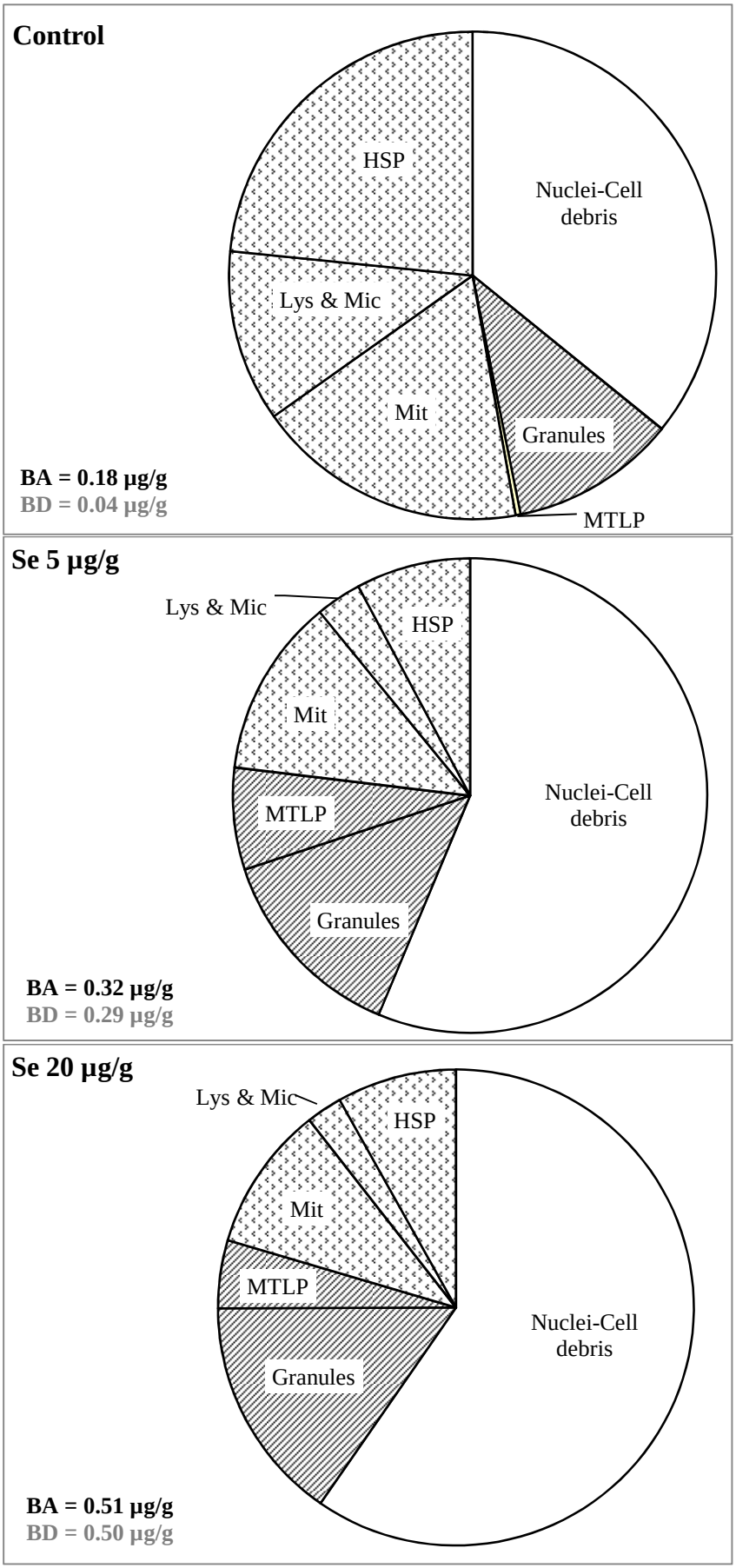
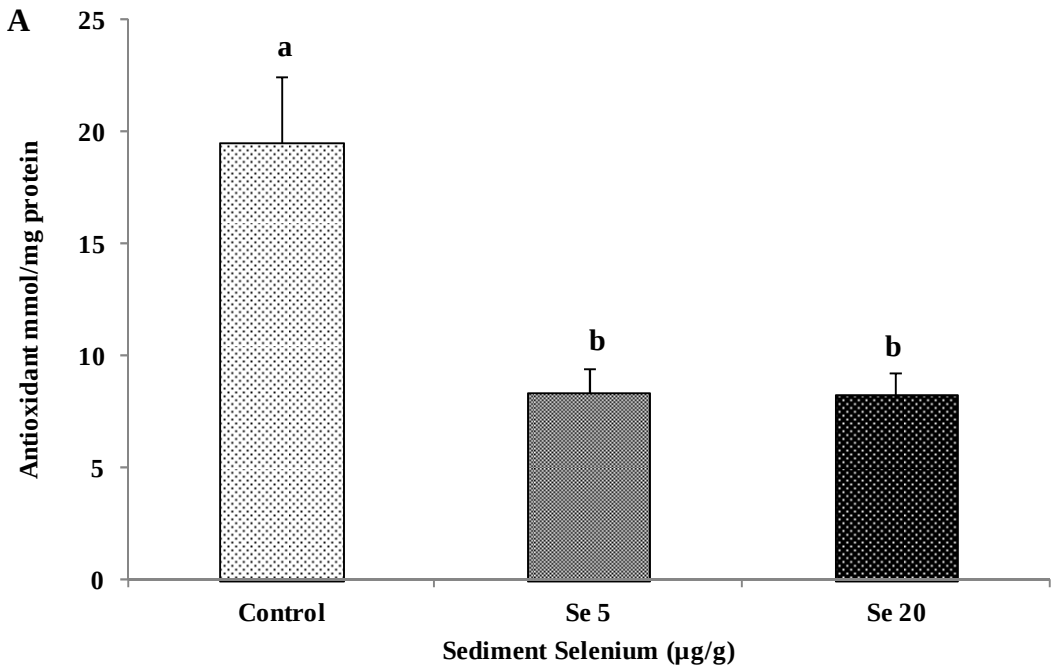
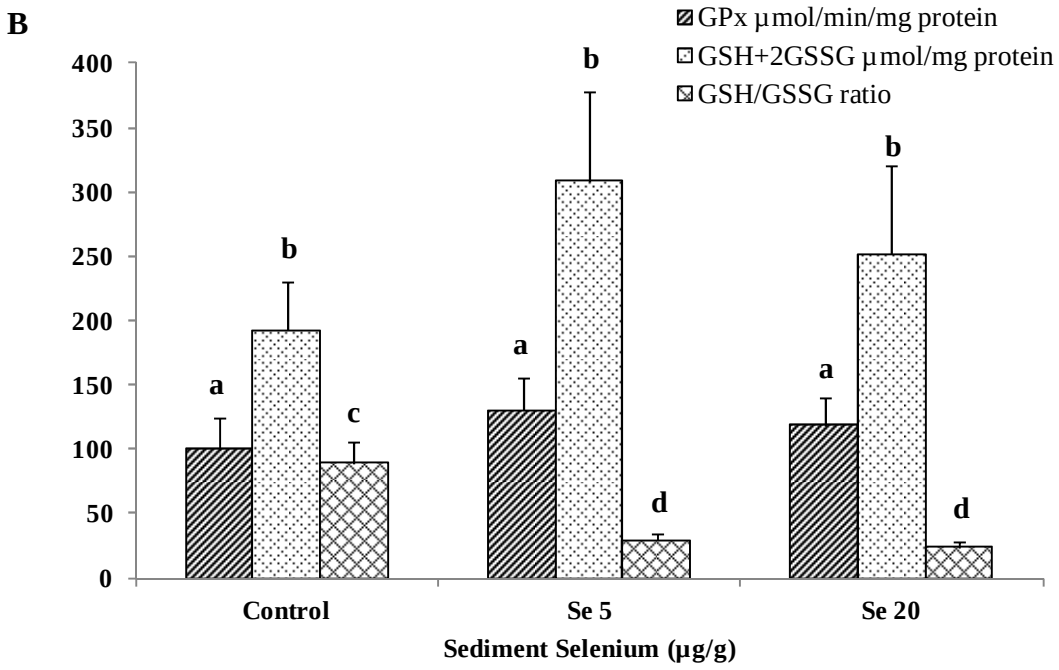
Figure 2


Control
HSP
Nuclei-Cell debris
Lys & Mic
Mit
Granules
MTLP
BA = 0.18 µg/g
BD = 0.04 µg/g
Se 5 µg/g
Lys & Mic
HSP
Mit
MTLP
Nuclei-Cell debris
Granules
BA = 0.32 µg/g
BD = 0.29 µg/g
Se 20 µg/g
Lys & Mic
HSP
Mit
MTLP
Nuclei-Cell debris
Granules
BA = 0.51 µg/g
BD = 0.50 µg/g

1031 **Figure 3**





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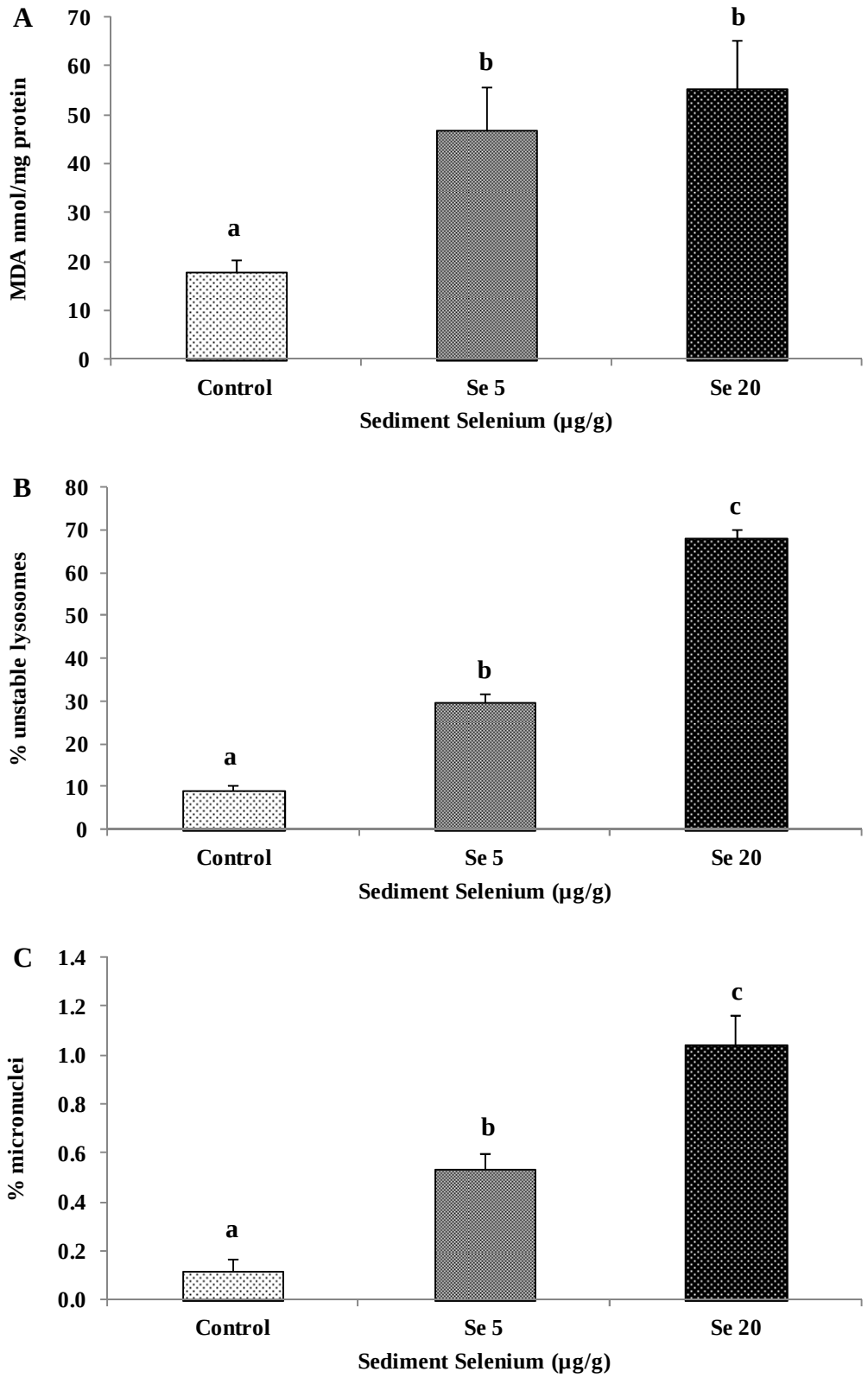
1034 **Figure 4**



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